THE ROYAL SOCIETY

Restoring avian wing digits

Neda Nikbakht and John C. McLachlan

University of St Andrews, School of Biological and Medical Sciences, Bute Building, St Andrews KY16 9TS, UK

The precise identification of the digits of the avian wing is of importance in evolutionary studies. If the digits are numbered two, three and four, this has been taken to suggest that birds are not descended directly from dinosaurs. If the digits are numbered one, two and three, dinosaur origins become more plausible. Studies of the development of the avian wing have failed to resolve this dilemma. However, in some instances, it is possible to deduce information about evolutionary morphologies by manipulating development experimentally. We grafted beads loaded with fibroblast growth factor 4 into the distal tip of chick wing buds at times when the apical ectodermal ridge is regressing. The consequence was that the cartilage structure conventionally labelled 'element 5' increased dramatically in size and acquired a digit-like morphology in some instances. Corresponding changes in soft tissue morphology were also observed. We conclude that it may be possible to resolve the issue of avian digit homology by the induction of experimental atavisms of this kind.

Keywords: fibroblast growth factor; embryonic; avian; limb; apical ectodermal ridge; dinosaur

1. INTRODUCTION

The apical ectodermal ridge (AER) is essential for vertebrate limb outgrowth and distalization (Summerbell 1974). Its action can be replaced by the implantation of heparin arcylic sulphate beads previously soaked in fibroblast growth factor (FGF)-4 (Niswander 1993). Purified FGF-8 protein has been shown to rescue limb bud outgrowth in mouse limbs lacking an AER, but has failed to maintain the expression of sonic hedgehog gene (*shh*) (Mahmood *et al*. 1995). In the chick, it has been shown that FGF-8 can replace the AER to maintain *shh* expression and outgrowth and patterning of the limb bud (Vogel *et al*. 1996). FGF-4 beads can also replace limb initiation by the AER, in that they can induce the outgrowth of additional limbs from the flank (Cohn 1995). Normally, the AER regresses at stage 26 (Summerbell 1974) (staging according to Hamburger & Hamilton 1992). Our intention was to observe the consequences of prolonging AER activity in development, with particular regard to the evolutionary implications. This approach of inducing experimental atavisms has previously been successfully employed in studies of the reappearance of the distal articulation of the fibula in the avian leg (Muller 1989). Spontaneous limb atavisms, such as the reappearance of elements of whale hind limbs (Hall 1984), or the reappearance of claws in the wings of the domestic hen (Cole 1967), demonstrate that the information for limb morphologies may become suppressed but remain present during the course of evolution.

The development of the cartilaginous elements of the avian wing has been extensively studied in the hope of resolving the problem of the homology of the digits. However, these studies have not produced a universally accepted answer; indeed workers have drawn opposite conclusions from their studies, perhaps because limb morphology has often been viewed in the light of existing prejudices on evolutionary history. To define avian digit homology, developmental biologists assume the conservation of embryonic patterning, while the palaeontologists use phylogenetic systematics and thus group birds with theropod dinosaurs, identifying the digits as one, two and three (Sereno & Novas 1992). Recently, it has been suggested, by comparison with crocodilians and reptiles, that the cartilaginous pattern is consistent with the identification of the elements as two, three and four (Burke & Feduccia 1997). Studies of forelimb development in turtles and alligators have shown that the primary axis unequivocally gives rise distally to digit four; this serves as a potential marker for digit identity and would assign the digit of the bird wing as two, three and four (Burke & Feduccia 1997). The reality is, however, that studies of the existing elements will probably never resolve this argument, since the information present is too scanty.

In addition to the digits conventionally numbered two, three and four, there is another element in the digital array, variously identified as the metacarpal of digit five, or a rudimentary digit five (Hinchliffe & Griffiths 1983). Obviously, exact identification of this element (which to avoid unwarranted assumptions we will call 'element 5') would be of value in resolving avian digit homologies.

2. METHODS

(a) *Experimental manipulation of chick limbs*

Fertilized white hen's eggs were incubated at 38 °C until they reached stages 25–26. The membranes were teased away to expose the wing bud. One or two heparin acrylic beads (Sigma H5263) soaked in FGF-4, as described below, were inserted into the tip of the wing bud just beneath the regressing AER. The beads were held in place with sterile platinum wire (diameter 0.025 mm), bent to form a staple. The limbs were examined the next day, and the position of the bead(s) within the limb bud recorded. Embryos in which the bead had moved from the

Received 23 December 1998 *Accepted* 29 January 1999

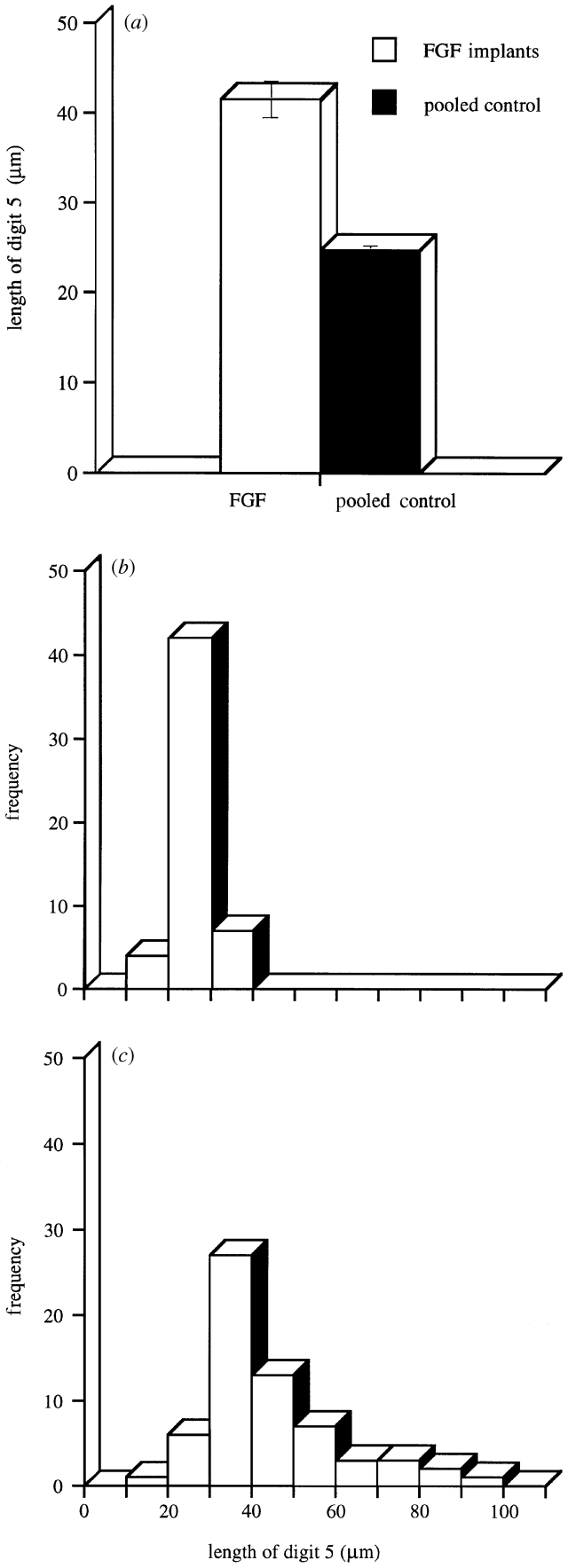

Figure 1. (*a*) Comparison of length of element 5 in FGF implanted limbs and controls. Error bars indicate standard errors. (*b*) Frequency distribution of lengths of element 5 in control limbs. (*c*) Frequency distribution of lengths of element 5 in FGF-implanted limbs.

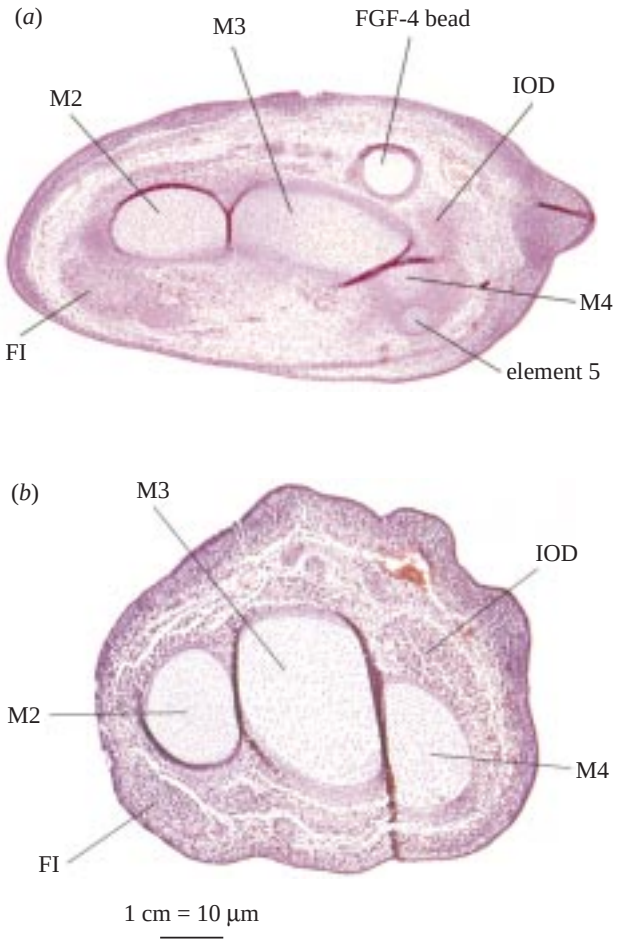

Figure 2. (*a*) Transverse section of FGF-treated limb at the most distal tip of element 5. (*b*) Transverse section of a control limb, corresponding to the same level as the experimental limb section. M4, metacarpal four; M3, metacarpal three; M2, metacarpal two; IOD, interosseus dorsalis; FI, flexor indicis. Scale: 1 cm = 10 μm.

original place or had been lost all together were discarded. Eggs were incubated for ten days, and the embryos were generally fixed in 5% trichloroacetic acid, stained with Alcian green and cleared in methyl Salicylate. After examination in whole mount, selected limbs were embedded in wax, sectioned and stained with haematoxylin–eosin according to standard procedures. Since tissue morphology is adversely affected by the whole-mount procedure, experimental and control limbs were also sectioned blind, without a whole-mount stage.

Control experiments took the form of mock operations without bead implants, implant of beads washed in phosphate buffered saline (PBS) and implant of beads soaked in PBS as below.

Limbs were photographed using a Wild dissecting microscope with a tube-mounted Olympus OM2 camera. Elements were measured directly from the negatives using a graticule scale.

(b) *Preparation of heparin beads*

A 2–3 μl drop containing 1 mg ml^{-1} recombinant FGF-4 (Sigma) was placed in the centre of a 35 mm plastic Petri dish, and surrounded with 20 drops of PBS each of 8 μl, to ensure humidification.

Heparin beads of diameter 200–250 μm were selected, washed in PBS and soaked for at least 1 h at room temperature in the

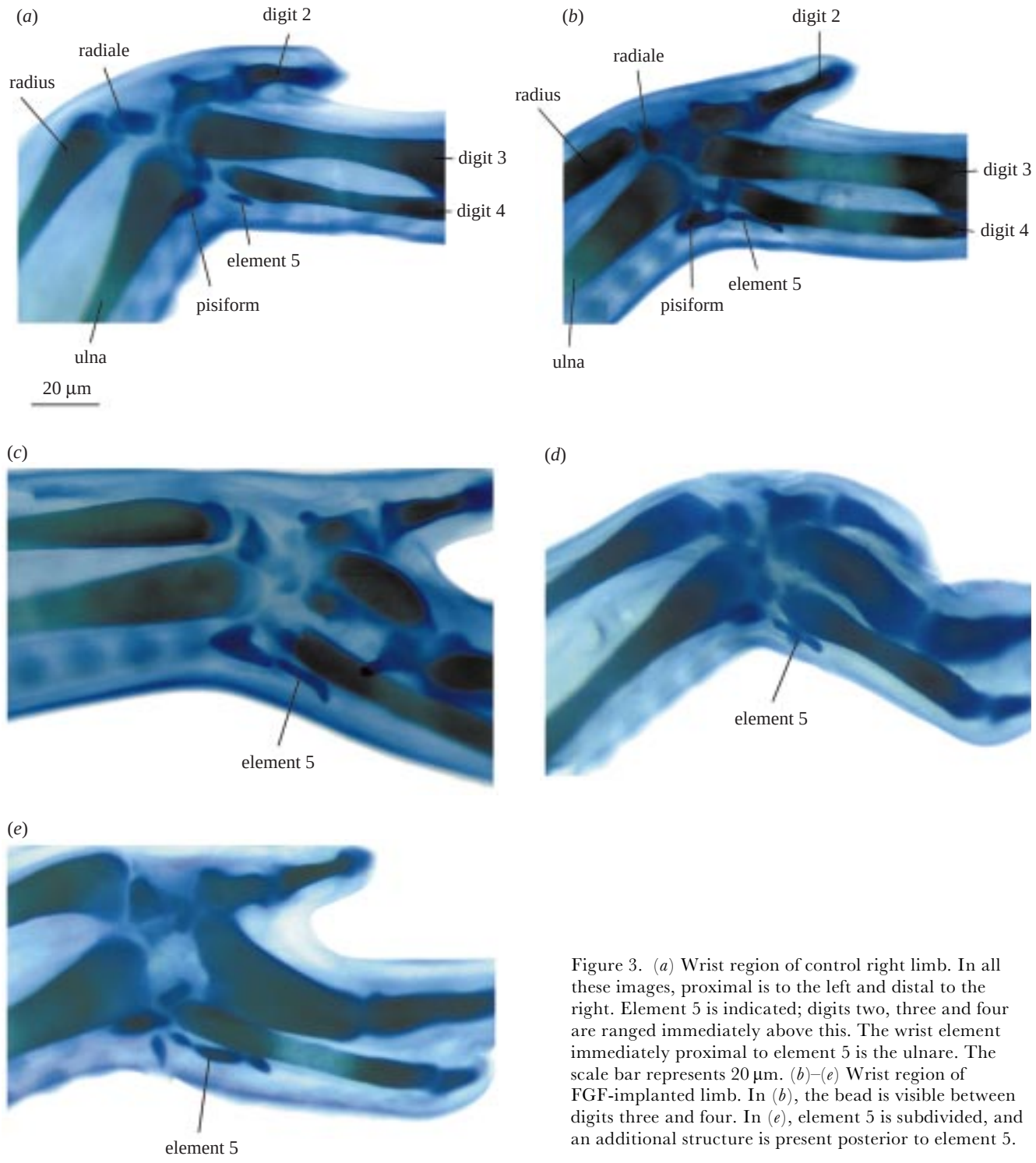

Figure 3. (*a*) Wrist region of control right limb. In all these images, proximal is to the left and distal to the right. Element 5 is indicated; digits two, three and four are ranged immediately above this. The wrist element immediately proximal to element 5 is the ulnare. The scale bar represents 20 µm. (*b*)–(*e*) Wrist region of FGF-implanted limb. In (*b*), the bead is visible between digits three and four. In (*e*), element 5 is subdivided, and an additional structure is present posterior to element 5.

FGF-4 solution prior to use within 3 h. Control beads were prepared in the same way, and soaked in PBS or used after washing.

(c) *Histology*

Limbs were sectioned transversely to the proximo-distal axis at 5 µm intervals and then stained with haematoxylin and eosin.

3. RESULTS

FGF beads were implanted in 150 chick limbs. Ninety-nine embryos survived, and out of these 63 survived the incubation period with beads in place. A total of 300 embryos were used for controls; 200 survived, and out of these 138 survived the incubation period with beads in place.

None of the control procedures affected the size or morphology of element 5. Results from all control limbs were therefore pooled. The FGF beads could disturb normal development in their immediate locality, in that elements were sometimes distorted or broadened and no real qualitative changes in the length of the three normal digits was observed.

The most consistent non-local consequence effect was a marked elongation of element 5 in FGF-treated limbs

(figure 1*a–c*, figure 3*a,b*) ($p < 0.001$ by *t*-test). The element was not markedly increased in width. The ulnar was generally also increased in size in experimental limbs. In some cases, element 5 appeared to show signs of further subdivision, suggesting that the element had given rise to phalangeal components (figure 3*c,d*). On rare occasions, an additional small cartilaginous structure was present posterior to element 5 (figure 3*e*). However, we would not wish to claim this as evidence that two elements can be provoked to develop from the caudal margin. This would require further development of the structures to a point where they are clearly identifiable as digits. No additional structures were identified anterior to digit two.

In the soft tissue of sectioned FGF-treated limbs, a number of changes had occurred. The FGF-treated limbs were compared with human limbs with regard to muscle and blood vessel morphology, since these provide an example of pentadactyl limb structure, and are exceptionally well-documented. In the chicks, muscle mass was increased distal to the wrist region: extra muscular and tendinous structures were visible but could not be identified by comparison with humans. Blood vessel pattern was also altered: the radial artery, which in chicks normally becomes insignificant at about the level of the tip of digit two (Levinsohn *et al*. 1984), and in humans supplies digits one and two, extended to the distal tip of digit three in FGF-treated limbs. There was a greater degree of branching of the ulnar artery at the level of the wrist. Figure 2*a* is a transverse section of a control chick limb stage 36 (ten days old) at the level where element 5 begins (distal tip). Figure 2*b* shows a section of an FGF-4 treated limb; element 5 is visible much earlier. By examining the serial sections made of both the experimental and the control, the approximate length of element 5 can be estimated, the sections were 5 µm. In the control, element 5 could only be seen in five sections, whereas in the experimentals, element 5 is visible in 20 sections.

4. DISCUSSION

The most important observation was that element 5 consistently elongated from proximal to distal in the form of a digit. This indicates that it is not a rudimentary carpal element as has been suggested. We believe that this element is a digit since it shows signs of phalangeal division. Several possible mechanisms may be responsible. For instance, the FGF implants may bring about persistence of AER beyond its natural life span, the increased vascularization to the wrist region may result in extended growth of element 5, or cell death may be inhibited.

We conclude that it is possible to induce experimental atavisms in chick digit morphology by prolonging the action of the apical ectodermal ridge by means of FGF implants under the ridge prior to regression. By these means, element 5 is shown to be a rudimentary digit. While we have not unequivocally identified this element as digit four or digit five, we believe that we have identified an experimental approach which is, in principle, capable of answering this question.

REFERENCES

Burke, A. C. & Feduccia, A. 1997 Developmental patterns and identification of homologies in the avian hand. *Science* **278**, 666–668.

Cohn, M. J., Izpisua-Belmonte, J. C., Abud, H., Heath, J. K. & Tickle, C. 1995 Fibroblast growth factors induce additional limb development from the flank of the chick embryos. *Cell* **80**, 739–746.

Cole, R. K. 1967 Ametapodia, a dominant mutation in the fowl. *J. Hered.* **58**, 141–146.

Hall, B. K. 1984 Developmental mechanisms underlying the formation of atavisms. *Biol. Rev.* **59**, 89–124.

Hamburger, V. & Hamilton, H. L. 1992 A series of normal stages in the development of chick embryos. *Dev. Dynam.* **195**, 231–272.

Hinchliffe, J. R. & Griffiths, P. J. 1983 The prechondrogenic patterns in tetrapod limb development and their phylogenetic significance. In *Development and evolution* (ed. B. C Goodwin, N. Holder & C. G. Wylie), pp 99–121. Cambridge University Press.

Levinsohn, E. M., David, S., Packard, J. R., West, E. M. & Hootnick, D. R. 1984 Arterial anatomy of chick embryo and hatchling. *Am. J. Anat.* **196**, 377–405.

Mahmood, R., Bresnick, J., Hornbruch, A., Mahony, C., Morton, N., Colquhoun, K., Martin, P., Lumsden, A., Dickinson, C. & Mason, I. 1995 A role for FGF-8 in the initiation and maintenance of vertebrate limb bug outgrowth. *Curr. Biol.* **5**, 797–806.

Muller, G. B. 1989 Ancestral patterns in bird limb development: a new look at Hampe's experiment. *J. Evol. Biol.* **2**, 31–47.

Niswander, L., Tickle, C., Vogel, A., Booth, I. & Martin, G. R. 1993 FGF-4 replaces the apical ectodermal ridge and directs outgrowth and patterning of the limb. *Cell* **75**, 579–587.

Sereno, P. C. & Novas, F. E. 1992 The complete skull and skeleton of an early dinosaur. *Science* **258**, 1137–1140.

Summerbell, D. 1974 A quantitative analysis of the effect of excision of AER from the chick limb-bud. *J. Embryol. Exp. Morph.* **32**, 651–660.

Vogel, A., Rodriguez, C. & Izpisua-Belmonte, J. 1996 Involvement of FGF-8 in initiation, outgrowth and patterning of the vertebrate limb. *Development* **122**, 1737–1750.